Phosphodependent Recruitment of Bub1 and Bub3 to Spc7/KNL1 by Mph1 Kinase Maintains the Spindle Checkpoint

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Report

Phosphodependent Recruitment of Bub1 and Bub3 to Spc7/KNL1 by Mph1 Kinase Maintains the Spindle Checkpoint

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Summary

The spindle assembly checkpoint (SAC) is the major surveillance system that ensures that sister chromatids do not separate until all chromosomes are correctly bioriented during mitosis. Components of the checkpoint include Mad1, Mad2, Mad3 (BubR1), Bub3, and the kinases Bub1, Mph1 (Mps1), and Aurora B [1]. Checkpoint proteins are recruited to kinetochores when individual kinetochores are not bound to spindle microtubules or not under tension [2–5]. Kinetochore association of Mad2 causes it to undergo a conformational change, which promotes its association to Mad3 and Cdc20 to form the mitotic checkpoint complex (MCC). The MCC inhibits the anaphase-promoting complex/cyclosome (APC/C) until the checkpoint is satisfied. SAC silencing derepresses Cdc20-APC/C activity. This triggers the polyubiquitination of securin and cyclin, which promotes the dissolution of sister chromatid cohesion and mitotic progression [6–8]. We, and others, recently showed that association of PP1 to the Spc7/Spc105/KNL1 family of kinetochore proteins is necessary to stabilize microtubule-kinetochore attachments and silence the SAC [9–12]. We now report that phosphorylation of the conserved MELT motifs in Spc7 by Mph1 (Mps1) recruits Bub1 and Bub3 to the kinetochore and that this is required to maintain the SAC signal.

Results

Bub1 and BubR1 checkpoint proteins are thought to bind kinetochores through interaction with the N-terminal region of KNL1 [13, 14]. Crystal structures of complexes between the tetratricopeptide repeat (TPR) domains of Bub1 and BubR1 and related, but distinct, motifs in KNL1 (named KI1 and KI2, respectively) have been generated [15, 16]. However, mutations in the TPR domain of Bub1 that abrogate its association to the KI1 domain of KNL1 do not block its association with kinetochores [16]. For this reason, the role of KNL1 in the kinetochore association of Bub1 and BubR1 has been brought into question. Mutation of a region of Bub1 (known as the GLEBS domain) that mediates its interaction with Bub3 prevents its association with kinetochores [16]. Although this agrees with a previous report, the binding site for Bub3 at kinetochores remains unknown [4]. In fission yeast, the Bub1 and Bub3 checkpoint proteins form a complex that binds to kinetochores during prometaphase and metaphase [17, 18]. Kinetochore association of Bub1 and Bub3 is dependent on the presence of both proteins and on Mph1 (Mps1) kinase [18–21]. Indeed, ectopic targeting of Mph1 to the outer kinetochore causes Bub1 to bind kinetochores throughout the cell cycle in checkpoint-deficient cells [22]. In this study, we examine the mechanism by which Mph1 directs the association of Bub1 and Bub3 to kinetochores.

Mph1 Kinase Phosphorylates Spc7

Bub1 fails to form discrete foci during mitosis in either *Δmph1* or *mph1(D459A)* mutants (which are defective for kinase activity [23]), indicating that catalytic activity of Mph1 is required to promote kinetochore association of Bub1 (Figure 1A). To examine whether Mph1 phosphorylates Spc7 to promote kinetochore association of Bub1, we tested whether Mph1 phosphorylates Spc7 in vitro. The N-terminal half of Spc7 (residues 1–666) was purified from bacteria and incubated in the presence of wild-type (WT) or catalytically inactive Mph1. We find that Mph1 phosphorylates Spc7 in vitro on two threonine residues (T453 and T507), which are part of a repetitive motif containing the expanded consensus sequence [M/I][E/D/N][I/L/M][S/T] (termed the MELT motif) that is present in all members of the Spc7/KNL1 family [24] (Figures 1B–1D; see also Figure S1A available online). Phosphoproteomic analysis has revealed that the threonine residues of two MELT motifs in mammalian KNL1 are phosphorylated in vivo [25, 26]. Although mutation of Spc7-T453 or Spc7-T507 alone did not substantially reduce phosphorylation of Spc7 by Mph1 in vitro, mutation of all nine threonine residues of the MELT motifs (Spc7-9TA) reduced Mph1-dependent phosphorylation of Spc7 by 63.5% (Figure S1B; data not shown). These results indicate that Mph1 phosphorylates one or more threonine residues in the MELT motifs of Spc7.

Phosphorylation of Spc7 Promotes Its Association to Bub1

To examine whether phosphorylation of Spc7 MELT motifs by Mph1 influences its association to Bub1, we constructed *spc7-9TA* and *spc7-9TE* mutants, in which the threonine residues of all nine Spc7 MELT motifs were mutated to either alanine or glutamic acid. Bacterially expressed Spc7, Spc7-9TA, and Spc7-9TE fusions proteins were incubated in extracts of *bub1-SZZ* cells. We find that Spc7-9TE, but neither WT Spc7 nor Spc7-9TA, fusion proteins efficiently precipitate Bub1 from cell extracts (Figure 1E). Additionally, we constructed *spc7-9MA* and *spc7-9MA,9TA* mutants, in which the methionine residues of all nine MELT motifs were mutated to alanine. When expressed from the endogenous promoter, all of these mutant Spc7 proteins localize to the kinetochore, are expressed at comparable levels to WT cells, and rescue viability in the absence of endogenous Spc7 (Figures S1C and S1D). We also find that the Spc7-9TE-GFP, but not WT Spc7-GFP, protein coimmunoprecipitates with Bub1 from extracts of log phase cells (Figure 1F). Together these data suggest that Mph1 phosphorylates Spc7 on MELT motifs to stimulate its association with Bub1 both in vitro and in vivo.

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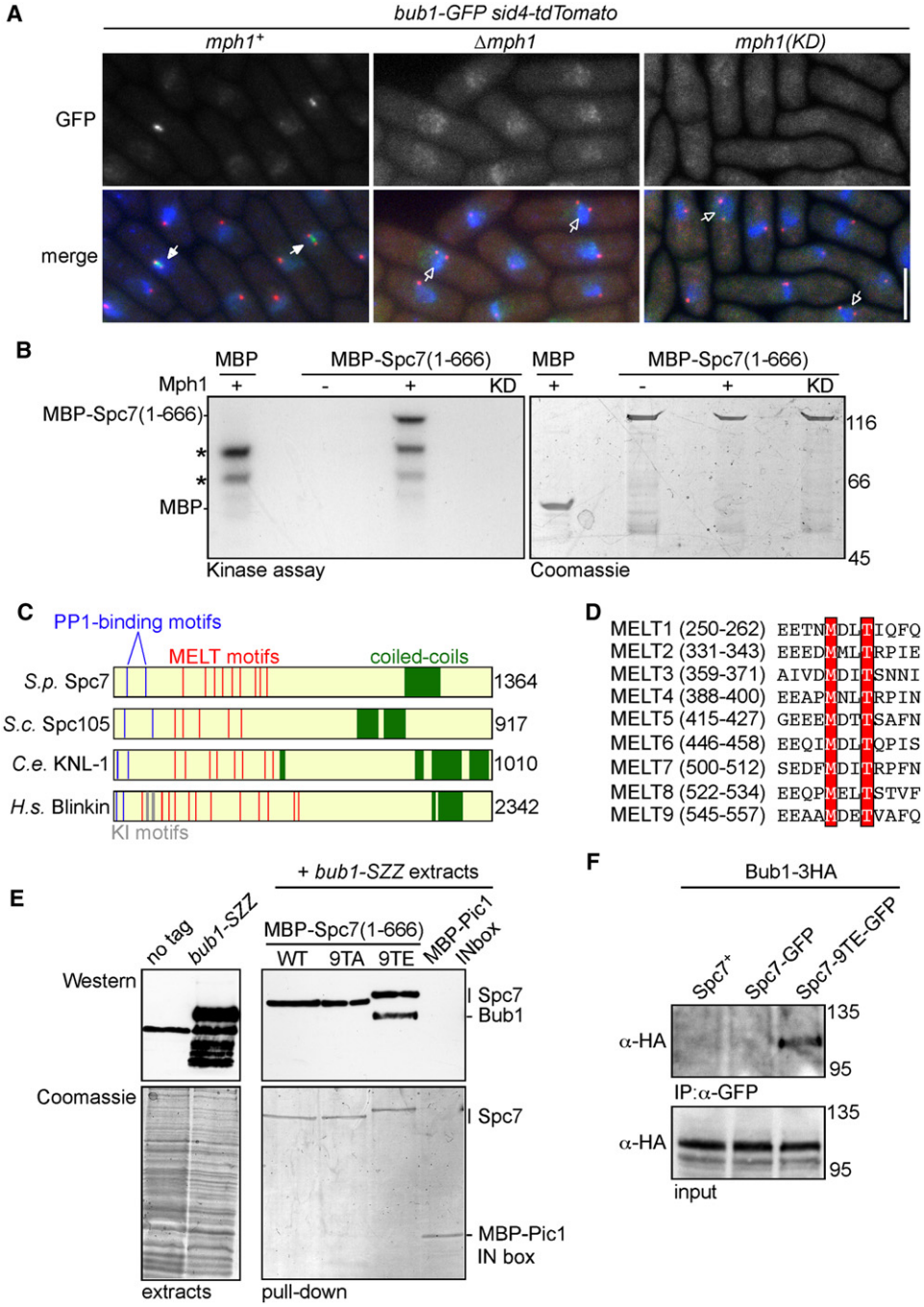

Figure 1. Mph1 Kinase Phosphorylates MELT Motifs in Spc7 to Promote Association of Spc7 with Bub1

(A) Log phase cultures of *bub1-GFP sid4-tdTomato* cells WT (*mph1*$^+$), lacking Mph1 (*Δmph1*), or defective in Mph1 kinase activity (*mph1[KD]*) were fixed and imaged. Representative images are shown. Cells with mitotic spindles less than 2.5 μm exhibiting localized Bub1-GFP (closed arrowheads) or lacking Bub1-GFP localization (open arrowheads) are highlighted. Scale bar represents 5 μm.

(B) Mph1 phosphorylates Spc7 in vitro. Mph1 kinase or catalytically inactive Mph1 (KD) was incubated with MBP or MBP-Spc7 (1–666) fusion protein. Kinase assay (left panel) and Coomassie stained gel of input proteins (right panel) are shown. Asterisks indicate Mph1 autophosphorylation.

(C) Domain architecture of fission yeast Spc7 and its homologs in budding yeast (*S.c.* Spc105), worm (*C.e.* KNL1), and human (*H.s.* blinkin). PP1-binding sites (blue), KI motifs (gray), MELT motifs (red), and the coiled-coil kinetochore-binding domain (green) are shown.

(D) Protein alignments of the nine MELT motifs in Spc7. Invariant methionine and threonine residues are highlighted in red.

(E) Spc7-9TE interacts with Bub1 in vitro. MBP-Spc7 (WT), MBP-Spc7-9TA (9TA), MBP-Spc7-9TE (9TE), or MBP-Pic1-INbox fusion proteins were incubated in extracts of untagged WT (no tag) or *bub1-SZZ* cells. Interacting proteins were precipitated on amylose beads, separated by SDS-PAGE, and subjected to western blot with anti-PAP antibody. Note that the anti-PAP antibody cross-reacts with the MBP-Spc7 proteins.

(F) Spc7-9TE interacts with Bub1 in vivo. Extracts from log phase *bub1-3HA spc7*$^+$, *bub1-3HA spc7-GFP*, or *bub1-3HA spc7-9TE-GFP* cells were prepared. Proteins were immunoprecipitated with anti-GFP antibodies, separated by SDS-PAGE, and subjected to western blot with anti-HA antibodies.

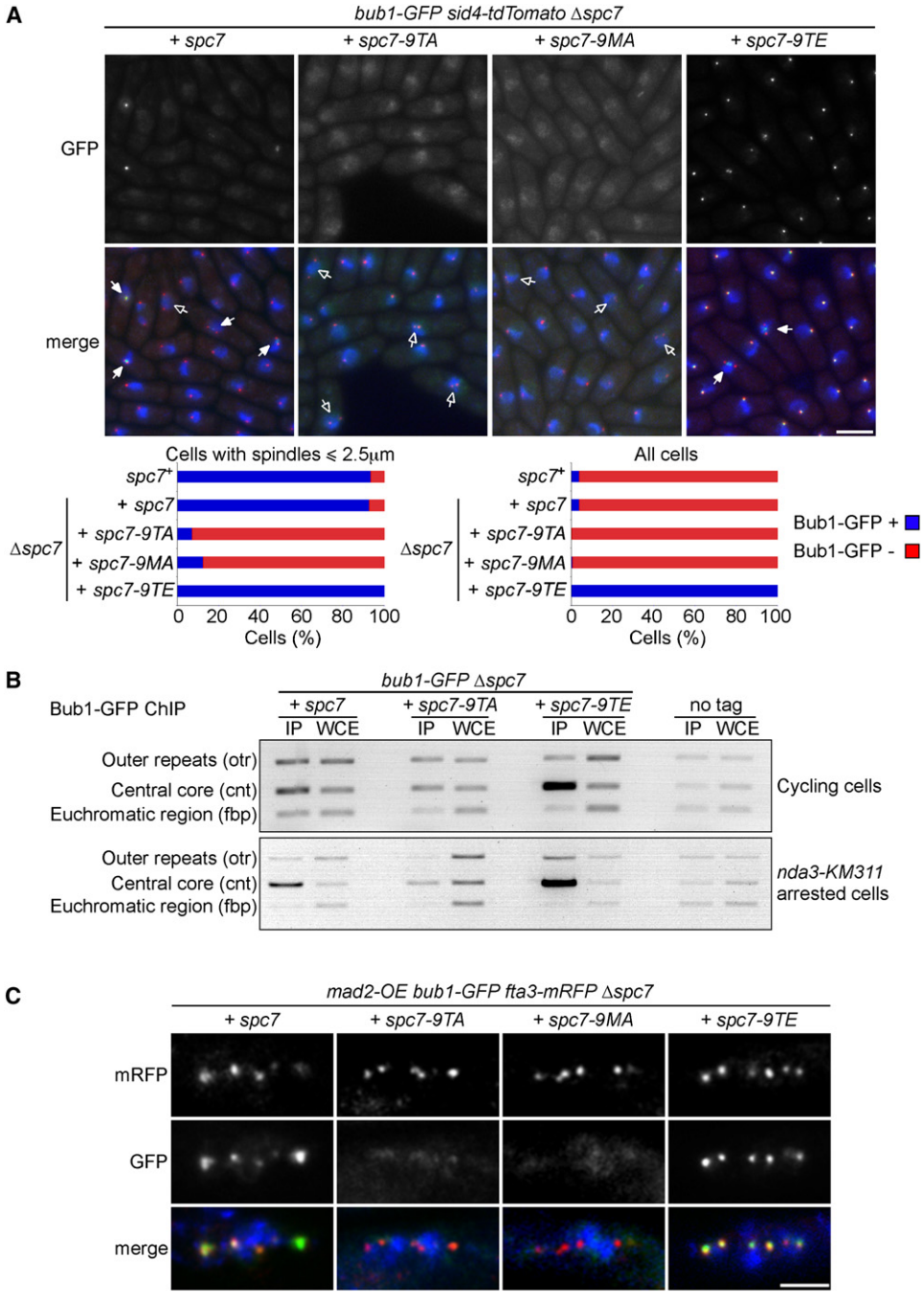

Figure 2. Phosphorylation of the MELT Motifs in Spc7 Recruits Bub1 to Kinetochores

(A) Log phase cultures of *Δspc7 spc7+*, *Δspc7 spc7-9TA*, *Δspc7 spc7-9MA*, and *Δspc7 spc7-9TE* expressing *bub1-GFP sid4-tdTomato* were fixed. The percentage of preanaphase mitotic cells (n = 3; left panels) or all cells (n = 3; right panels) with Bub1 foci (closed arrowheads, blue bars) or lacking foci (open arrowheads, red bars) was assessed. Scale bar represents 5 μm.

(B) Log phase or metaphase arrested cultures of *Δspc7 spc7+*, *Δspc7 spc7-9TA*, and *Δspc7 spc7-9TE* cells expressing *nda3-KM311 bub1-GFP* or *nda3-KM311* cells (no tag) were fixed and subjected to ChIP analysis with anti-GFP antibodies. The immunoprecipitated DNA was amplified by PCR using primers specific to the central core (cnt), which is the site of kinetochore assembly, and control primers that amplify a noncentromeric, euchromatic negative control (fbp) or centromeric outer repeats (otr). Representative PCRs are shown for each tagged protein, as well as the untagged negative control.

(C) Cultures of *Δspc7 spc7+*, *Δspc7 spc7-9TA*, *Δspc7 spc7-9MA*, and *Δspc7 spc7-9TE* expressing *bub1-GFP fta3-mRFP pREP3x-mad2* were incubated in medium lacking thiamine for 18 hr to induce Mad2 overexpression. Cells were fixed and imaged. Representative images are shown. Scale bar represents 2 μm.

Phosphorylation of Spc7 Recruits the Bub1-Bub3 Complex to Kinetochores

These results persuaded us to examine the influence of Spc7 phosphorylation on cell-cycle-dependent localization of Bub1. We find that Bub1 localizes between separated spindle poles in control and *spc7-9TE* cells during early mitosis, but is undetectable in either *spc7-9TA* or *spc7-9MA* mutants during mitosis or at any other stage of the cell cycle (Figure 2A).

Strikingly, Bub1 colocalizes with spindle poles throughout the cell cycle in *spc7-9TE*, but not in WT, cells (Figures 2A). To verify this, we performed chromatin immunoprecipitation (ChIP) in cycling and metaphase arrested cultures. Whereas Bub1 binds to centromeric DNA only during mitosis in WT cells, Bub1 binds strongly to centromeric DNA even in cycling *spc7-9TE* cells (Figure 2B). Notably, association of Bub1 to centromeric DNA is substantially reduced, but not completely abolished, in metaphase arrested *spc7-9TA* cells (Figure 2B). To confirm this localization, we monitored Bub1-GFP localization in *fta3-mRFP* cells following overexpression of Mad2, which arrests cells in metaphase by hyperactivation of the SAC [27]. Fta3 is a component of the Mis6 complex that binds the kinetochore throughout the cell cycle [28]. Although Bub1 colocalizes with kinetochores in WT and *spc7-9TE* cells, we were unable to detect Bub1 at kinetochores in metaphase arrested *spc7-9TA* or *spc7-9MA* cells (Figure 2C). These data suggest that Mph1 phosphorylates the MELT motifs in Spc7 to recruit Bub1 to kinetochores during early mitosis.

We next examined the dependency of Bub1 localization on Mph1 and Bub3. Bub1 colocalizes with kinetochores throughout the cell cycle in *mph1-D459A spc7-9TE* cells but not in *Δbub3 spc7-9TE* cells. The phosphomimetic *spc7-9TE* mutant therefore bypasses the requirement for Mph1 kinase activity to load Bub1 to kinetochores, but Bub1 loading still requires the presence of Bub3 (Figure 3A). Consistent with this, interaction of bacterially expressed Spc7-9TE with Bub1 in cell extracts requires Bub3, but not Mad3 (Figure S2A). Like Bub1, Bub3 localizes to the kinetochore throughout the cell cycle in *spc7-9TE* cells but fails to accumulate at kinetochores in mitotic *spc7-9TA* or *spc7-9MA* cells (Figure 3B). Moreover, association of Bub3 with kinetochores is dependent on Mph1 activity, and ectopic association of Bub3 to kinetochores in *spc7-9TE* cells bypasses the requirement for Mph1 (Figures S2B and S2C). However, Bub3 loading still requires Bub1 in these cells (Figure S2C). These data suggest that Mph1 phosphorylation of the MELT motifs in Spc7 recruits the Bub1-Bub3 complex to kinetochores. Notably, ectopic association of Bub1 and Bub3 to kinetochores in spc7-9TE cells does not by itself cause cell-cycle arrest. By contrast, when Mph1 is ectopically tethered to Ndc80, Mph1 promotes both association of Bub1 to kinetochores and SAC arrest [22]. One possibility is that, in this situation, Mph1 phosphorylates other components of the SAC, such as Mad1, Mad2, and Mad3, to directly activate the SAC, or destabilizes microtubule-kinetochore interactions to indirectly activate the SAC [23, 29].

Phosphorylation of Spc7 Is Required for Normal Recruitment of Mad1 and Mad2 to Kinetochores

We previously found that association of Mad1 and Mad2 to kinetochores is substantially reduced in cells lacking Bub3 [30]. This persuaded us to examine whether phosphorylation of Spc7 MELT motifs influences the localization of other SAC proteins. In WT cells, Mad1 and Mad2 form bright foci that colocalize with kinetochores in approximately 3% of cells. We find that localization of Mad1 and Mad2 to kinetochores is substantially diminished, but not completely abolished, in mitotic *spc7-9TA* and *spc7-9MA* cells (Figures S3A and S3B). Importantly, neither Mad1 nor Mad2 is ectopically recruited to kinetochores during interphase in *spc7-9TE* cells, indicating that ectopic recruitment of Bub1 and Bub3 to kinetochores is not sufficient to recruit Mad1 and Mad2, consistent with previous observations [22] (Figures S3A and S3B).

Spc7-MELT Mutants Have Severe Chromosome Segregation Defects

We next examined the effect of *spc7-MELT* mutants on chromosome segregation during mitosis. We find that *spc7-9TA*, *spc7-9MA*, and *spc7-9MA,9TA* mutants are acutely sensitive to thiabendazole (TBZ), a microtubule depolymerizing agent, and profoundly defective in maintenance of an artificial minichromosome, similar to that observed in the absence of Mph1 [31] (Figures 4A and 4B). By comparison, *spc7-9TE* mutants are slightly sensitive to TBZ and have no appreciable defect in chromosome segregation compared to WT cells (Figures 4A and 4B). Congruously, we find that *spc7-9TA*, *spc7-9MA*, and *spc7-9MA,9TA*, but not *spc7-9TE*, mutants are lethal in cells lacking Dis2 (type 1 phosphatase), Klp5 (kinesin-8), or Dis1 (XMAP215), all of which are required for accurate chromosome segregation (Table S1; [32–34]). Notably, both *spc7-9TA* and *spc7-9TE* mutants are synthetically lethal with *Δdam1* mutants, indicating that the *spc7-9TE* mutant is not completely WT (Table S1). Cells lacking Dam1, Dis2, Klp5, or Dis1 exhibit delayed anaphase onset and numerous synthetic lethal interactions with components of the SAC [34–38]. By contrast, none of the *spc7-MELT* mutants showed any synthetic interactions with cells lacking Bub1, Bub3, Mph1, Mad2, or Mad3 (Table S1). Moreover, the synthetic lethality between *spc7-MELT* mutants and cells lacking Dam1, Dis2, Klp5, or Dis1 is not reversed by simultaneously deleting Mad3, indicating that these synthetic lethalities are probably due to a defect in chromosome segregation rather than hyperactivation of the SAC (Table S1).

Spc7-MELT Mutants Are Defective in Maintenance of the Spindle Checkpoint

To examine the effect of *spc7-MELT* mutants on the timing of anaphase onset, we first measured the percentage of cells with spindle and spindle pole associated Cdc13 (cyclin B) in log phase populations. Both *spc7-9TA* and *spc7-9MA* mutants spend longer in prometaphase and metaphase than *spc7-9TE* or control cells. This is likely to be due to activation of the SAC caused by defects in kinetochore-microtubule attachment. Consistent with this hypothesis, we found that the delay in anaphase onset observed in these mutants is dependent on Mad3 (Figure 4C). To examine the efficiency of SAC signaling in *spc7-MELT* mutants, we assayed their SAC arrest in cells that carry the cold-sensitive *nda3-KM311* allele of the gene encoding β-tubulin [39]. Unfortunately, we were unable to generate *spc7-9TA nda3-KM311 cdc13-GFP* strains, so instead we utilized Nsk1-GFP as an anaphase marker. Nsk1 is a recently described substrate of Cdk1 kinase that binds spindle poles only during anaphase B when cyclin B is degraded and Nsk1 is dephosphorylated [35, 40]. Nsk1 localization is unaffected in *spc7-MELT* mutants in log phase cultures (Figure S4A). To quantify the efficiency of SAC signaling, we synchronized cells in early G2 using lactose gradients, then shifted to 18°C for 6 hr. Nsk1 localization was analyzed at 30 min intervals. The *nda3-KM311 spc7*$^{+}$ cells maintain a SAC arrest for 6 hr: no cells are observed with Nsk1-GFP at spindle poles. Using *Δmad3* as a checkpoint null mutant control, we saw a wave of cells with Nsk1-GFP at spindle poles. These peaked at 2.5 hr (~30% of the culture) and then the numbers dropped as cells entered the next cell cycle and Nsk1-GFP was no longer at spindle poles. By analyzing the area under this curve, it can be seen that, during the 6 hr time course, almost all of the *Δmad3* cells (>90%) have exited mitosis, as expected for a checkpoint mutant (Figure 4D).

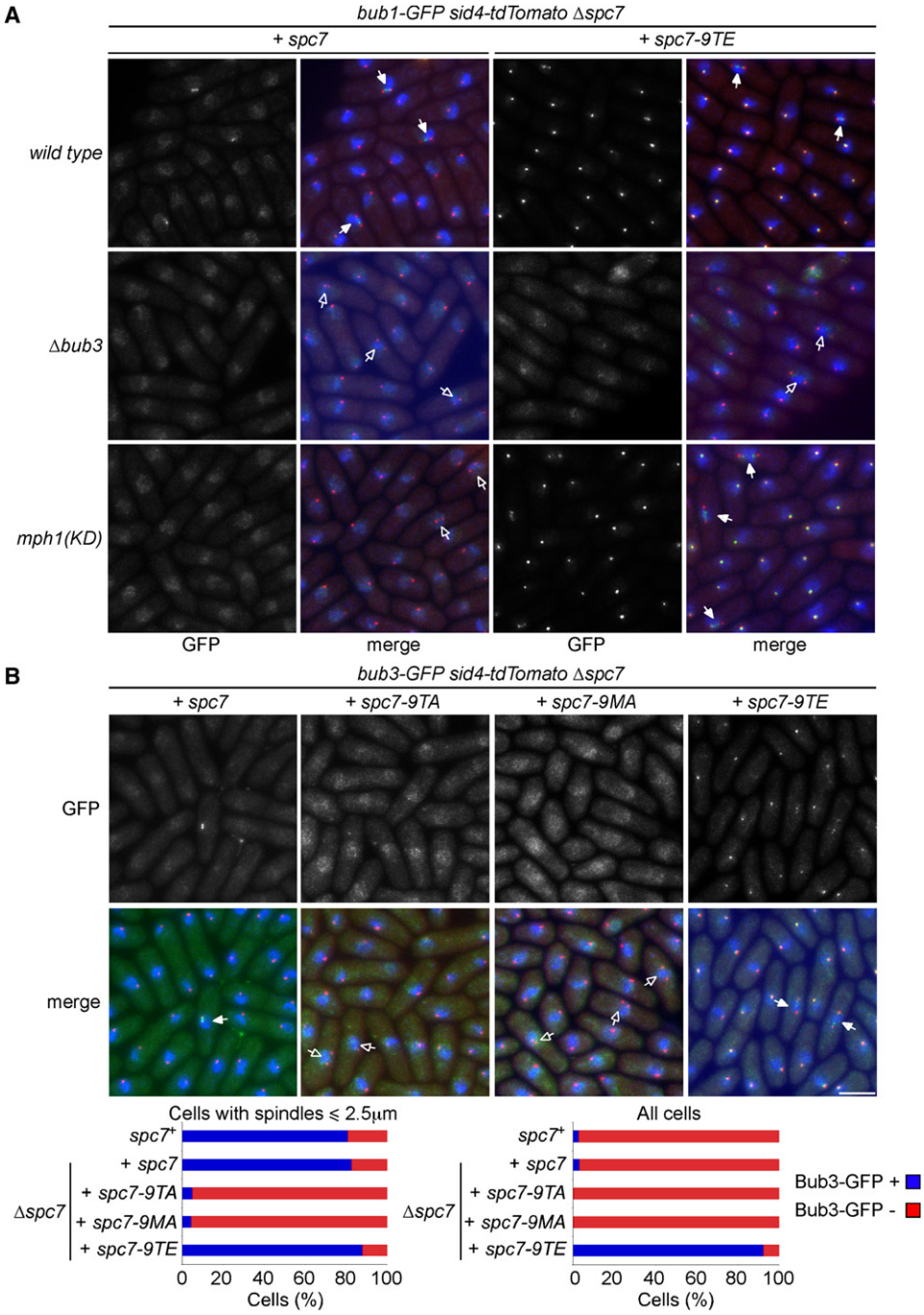

Figure 3. Mph1-Dependent Recruitment of Bub1 to Kinetochores Requires Bub3

(A) Log phase cultures of *bub1-GFP sid4-tdTomato Δspc7 spc7*$^{+}$ and *bub1-GFP sid4-tdTomato Δspc7 spc7-9TE* cells either WT (*wild-type*), lacking Bub3 (*Δbub3*), or defective in Mph1 kinase activity (*mph1[KD]*) were fixed and imaged. Cells with mitotic spindles less than 2.5 μm exhibiting localized Bub1-GFP (closed arrowheads) or lacking Bub1-GFP localization (open arrowheads) are highlighted. Scale bar represents 5 μm.

(B) Log phase cultures of *Δspc7 spc7*$^{+}$, *Δspc7 spc7-9TA*, *Δspc7 spc7-9MA*, and *Δspc7 spc7-9TE* expressing *bub3-GFP sid4-tdTomato* were fixed. The percentage of preanaphase mitotic cells (n = 3; left panels) or all cells (n = 3; right panels) with Bub3 foci (closed arrowheads, blue bars) or lacking foci (open arrowheads, red bars) was assessed. Scale bar represents 5 μm.

Nsk1-GFP does not appear at spindle poles at early time points in *spc7-9TA* mutants, indicating that phosphorylation of the MELT motifs is not required for the initial SAC response when microtubules are completely absent (2 hr time point, Figure 4D). However, at later time points, Nsk1 appears at spindle poles in ~10% of *spc7-9TA* cells (3–6 hr time points, Figure 4D) indicating that phosphorylation of the MELT motifs is required to maintain the SAC signal. Counting the area under this curve shows that >60% of these cells have failed to maintain the SAC arrest through the 6 hr time course. SAC proficiency is similarly affected in *spc7-9MA* cells, and to a lesser extent in *spc7-9TE* cells (Figure S4B). This checkpoint defect is stronger than that

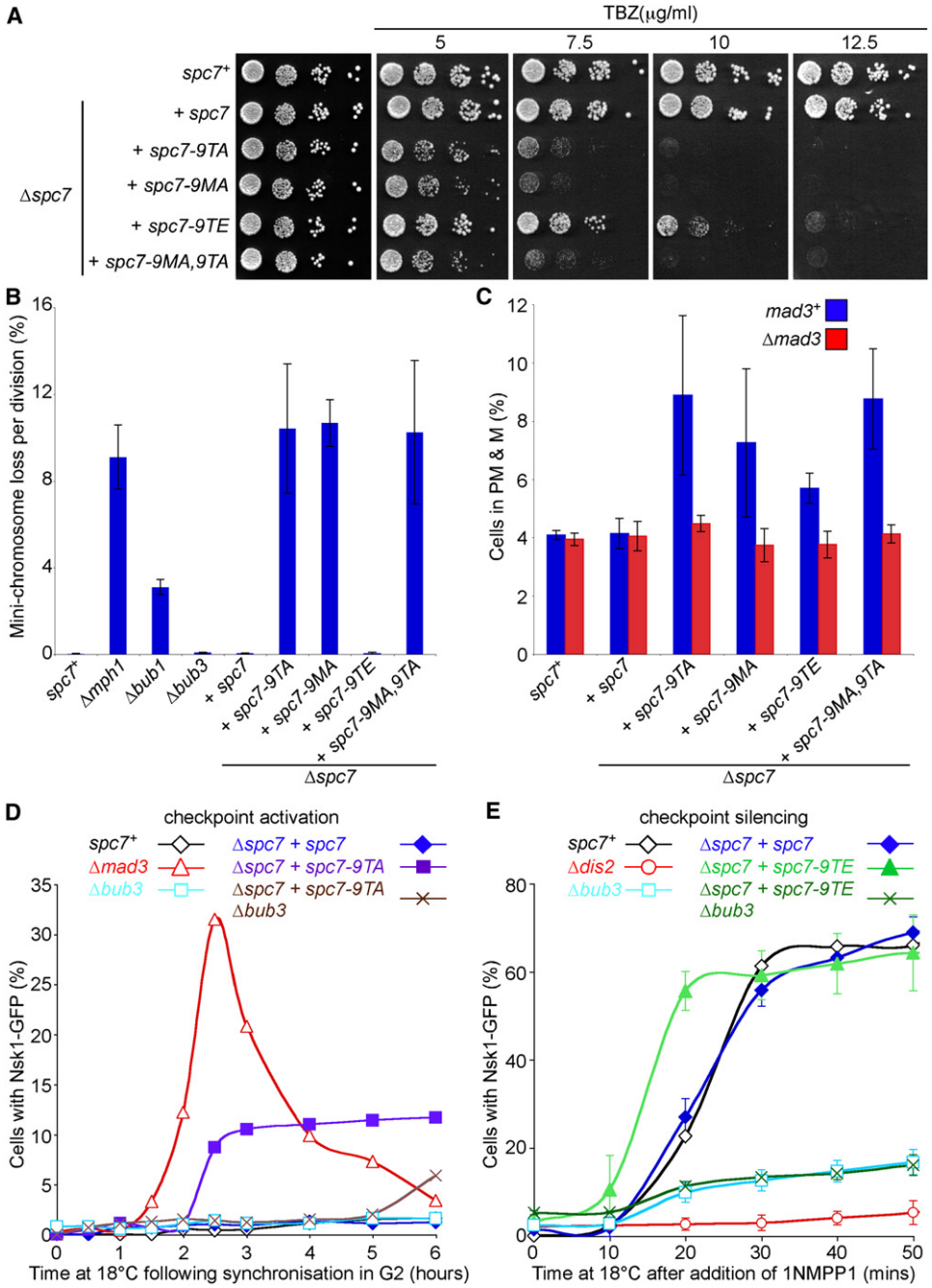

Figure 4. Phosphorylation of Spc7 MELT Motifs Is Required for Accurate Chromosome Segregation and Maintenance of the Spindle Checkpoint

(A) *spc7-MELT* mutants are sensitive to thiabendazole (TBZ). Serial dilutions of *wild-type* (*spc7*$^+$), *Δspc7 spc7*, *Δspc7 spc7-9TA*, *Δspc7 spc7-9MA*, *Δspc7 spc7-9TE*, and *Δspc7 spc7-9MA,9TA* cells were plated onto YEA (yeast extract with adenine) plates containing indicated TBZ concentrations and incubated for 3 days at 30°C.

(B) Loss of the Ch16 (*ade6-M216*) mini-chromosome was measured using a colony-sectoring assay. Error bars represent the SD of three independent experiments.

(C) Anaphase onset is profoundly delayed in *spc7-9TA, spc7-9MA* and *spc7-9MA,9TA* but only slightly delayed in *spc7-9TE* mutants. Log phase cultures of *wild-type* (*spc7*$^+$), *Δspc7 spc7*, *Δspc7 spc7-9TA*, *Δspc7 spc7-9MA*, *Δspc7 spc7-9TE*, and *Δspc7 spc7-9MA,9TA* cells, expressing *cdc13-GFP* in the presence (blue bars) or absence (red bars) of Mad3 were fixed and the percentage of cells with Cdc13 on spindles and separated spindle pole bodies was assessed. Error bars represent the SD from three independent experiments.

(D) Phosphorylation of Spc7 MELT motifs is required for maintenance of the spindle checkpoint. Log phase cultures of *wild-type* (*spc7*$^+$), *Δmad3*, *Δbub3*, *Δspc7 spc7*, *Δspc7 spc7-9TA*, and *Δspc7 spc7-9TA Δbub3* cells expressing *nda3-KM311 ark1-as3 nsk1-GFP* were synchronized in early G2 by lactose gradient centrifugation and incubated at 18°C for the times indicated. The cells were fixed, and the percentage of cells with spindle pole associated Nsk1 was assessed.

(E) Ectopic recruitment of Bub1 and Bub3 to Spc7 aids spindle checkpoint silencing. Log phase cultures of *wild-type* (*spc7*$^+$), *Δdis2*, *Δbub3*, *Δspc7 spc7*, *Δspc7 spc7-9TE*, and *Δspc7 spc7-9TE Δbub3* cells expressing *nda3-KM311 ark1-as3 nsk1-GFP* were synchronized in prometaphase by incubating at 18°C for 6 hr, and 5 μM 1NMPP1 was added. At the times indicated, the cells were fixed and the percentage of cells with spindle pole associated Nsk1 was assessed. Error bars represent the SD from three independent experiments.

observed in *Δbub3* fission yeast cells, where neither Bub1 nor Mad3 can be recruited to kinetochores, yet the SAC remains robust (Figure 4D; [30, 41]). This creates an apparent paradox: why should mutation of the kinetochore binding site for Bub1-Bub3 cause more of a SAC defect than completely abolishing Bub1 recruitment to kinetochores (in *Δbub3*)? Surprisingly, when *spc7-9TA* was combined with *Δbub3*, the SAC response was much improved, although this did not suppress the chromosome missegregation defects of *spc7-9TA* (Figure 4D; data not shown). This demonstrates that the SAC defect observed in *spc7-9TA* is not due to reduced Bub1-Bub3 recruitment at kinetochores, but it suggests that when the Bub1-Bub3 complex is not bound to Spc7, it acts as a dominant-negative factor that prevents maintenance of the SAC signal. The precise explanation of this effect requires further analysis.

Enhanced Recruitment of Bub1-Bub3 to Spc7 Aids Spindle Checkpoint Silencing

We previously showed that *Δbub3* mutants, where Bub1 and Mad3 are entirely absent from kinetochores, have SAC silencing defects [30], so we were keen to analyze SAC silencing in *spc7-MELT* mutants. Unfortunately, we were unable to construct the necessary *spc7-9TA* or *spc7-9MA* strains, but we have analyzed the *spc7-9TE* silencing phenotype. To do this, we monitored Nsk1 association to spindle poles following chemical inactivation of Ark1 (Aurora B) in mitotically arrested *nda3-KM311 ark1-as3 nsk1-GFP* cells. In this assay, the SAC is inactivated by addition of 1NMPP1, which selectively inhibits analog-sensitive Ark1 [42], thus promoting silencing (or override) of the SAC signal. In this situation, $PP1^{Dis2}$ is essential for dissociation of Mad2 and Mad3 from the APC/C and for activation of the APC/C complex, which triggers cyclin B destruction and appearance of Nsk1 on spindle poles (Figure 4E; [35, 43]). We find that, upon addition of 1NMPP1, Nsk1 appears more rapidly on spindle poles in *spc7-9TE* cells than in WT cells (Figure 4E). Likewise, we also observe more rapid destruction of Cdc13 (cyclin B) in metaphase arrested *nda3-KM311 ark1-as3 cdc13-GFP spc7-9TE* cells upon addition of 1NMPP1 than in control cells (Figure S4C). Importantly, the rapid appearance of Nsk1 on spindle poles in *spc7-9TE* cells is completely abolished in the absence of Bub3 (Figure 4E). These results suggest that enhanced recruitment of Bub1 and Bub3 to the kinetochore in *spc7-9TE* cells promotes silencing of the SAC to such an extent that it becomes more efficient than in WT cells.

Discussion

At present, it is unclear whether Bub1 or Bub3 interact directly with phosphorylated MELT motifs in Spc7/KNL1. Bub3 contains seven WD40 repeats that are arranged in a radial pattern to form a β-propeller, a three-dimensional structure that mediates protein-protein interactions [44]. Some proteins containing WD40 domains, such as Cdc4, only interact with phosphorylated target proteins. Indeed Sic1, an inhibitor of cyclin B/Cdk1 in budding yeast, is only recognized and ubiquitinylated by SCF^{Cdc4} when phosphorylated on multiple sites by G1 and S phase cyclin/Cdk1 complexes [45]. In the same manner, Bub3 may only interact with Spc7/KNL1 that has been multiply phosphorylated to recruit Bub1. If this is the case, we predict that the methionine of the MELT motif is also essential for Bub3 binding because *spc7-9TA*, *spc7-9MA*, and *spc7-9TA,9MA* mutants have indistinguishable phenotypes. However, we observe weak binding of Bub1 to centromeric DNA in metaphase arrested *spc7-9TA* cells, indicating that Bub1 may form additional contacts with Spc7 that contribute to the overall stability of the Spc7-Bub1-Bub3 complex. This may be analogous to the interaction observed between the TPR domains of Bub1 and the KI1 domain of KNL1, although the KI motifs do not appear to be conserved in fission yeast Spc7 [13, 14, 16]. Alternatively, because Mph1 can phosphorylate other sites in Spc7-9TA in vitro, Mph1 may promote Bub1 binding through a mechanism that does not involve phosphorylation of the MELT motifs.

It is important to note that *spc7-9TA*, *spc7-9MA*, and *spc7-9TA,9MA* mutants missegregate chromosomes at high frequency, whereas cells lacking Bub3 are only marginally more defective than WT [41] (Figure 4B). Thus, phosphorylation of the MELT motifs is necessary for accurate chromosome segregation independently of, or in addition to, recruitment of Bub1 and Bub3. Potentially, MELT motifs could bind an unidentified factor required for chromosome segregation. Alternatively, phosphorylation of the MELT motifs may influence the dynamic architecture of the outer kinetochore KMN complex during mitosis, and thus reduce the ability of kinetochores to interact with microtubules. For example, MELT phosphorylation may be required to position $PP1^{Dis2}$ bound to the N terminus of Spc7 near its substrates so that it can stabilize microtubule-kinetochore interactions. Two studies have recently demonstrated that intrakinetochore stretch is necessary and sufficient to satisfy the SAC, although it is unknown how this stretching is sensed [46, 47]. Because *spc7-9TA* and *spc7-9MA* mutants are profoundly defective in chromosome segregation and fail to accumulate SAC components at kinetochores, it is tempting to speculate that the repetitive MELT motifs in Spc7/KNL1 may act as a quantitative sensor of intrakinetochore stretch. Further experiments are needed to address this and other possibilities.

Notably, the accompanying paper by London et al. [48] in this issue of *Current Biology* indicates that Mps1 is the major kinase that associates with kinetochores purified from budding yeast cells and, second, that phosphorylation of the MELT motifs in Spc105 (the budding yeast homolog of Spc7) by Mps1 is required for Bub1-Bub3 recruitment to kinetochores, suggesting that the mechanism targeting the Bub1-Bub3 complex to kinetochores is conserved [48]. Although Bub3 is not critical for SAC arrest in *S. pombe* [30, 41, 49], disruption of the binding sites for the Bub1-Bub3 complex on Spc7 causes a defect in maintenance of the SAC signal. Conversely, ectopic recruitment of the Bub1-Bub3 complex to Spc7 in *spc7-9TE* cells enhances SAC silencing, providing strong confirmation of our previous observations [30]. One possibility is that association of the Bub1-Bub3 complex to the MELT motifs in Spc7/KNL1 provides a docking site for the MCC to be dephosphorylated and inactivated by PP1, which binds the N terminus of Spc7/KNL1 [11, 12]. Although this model is attractive, it is probably too simplistic because at least one other pool of PP1, bound to Klp5/Klp6 complex (kinesin-8), is also required for efficient SAC silencing in fission yeast [11]. Clearly, identifying the PP1 targets required for SAC silencing and analyzing the function of the conserved MELT motifs in KNL1 for SAC signaling in vertebrate cells are important goals for the future.

Supplemental Information

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.03.051.

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